Phospholipid flippases and Sfk1 are essential for the retention of ergosterol in the plasma membrane

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RE: Manuscript #E20-11-0699
TITLE: Phospholipid flippases and Sfk1 are essential for the retention of ergosterol in the plasma membrane

Dear Dr. Kishimoto,
Your manuscript has now been seen by two experts in the field. They have several criticisms, which you can see by their extensive reports. My conclusion is that you have assembled a large quantity of data, most of it very good, but no single experiment validates your conclusions/hypothesis alone, which makes the presentation of your arguments very important. I think that a large part of the problem is in the presentation. Perhaps if it were more clearly and better organized reviewer 2 may have been more convinced.
The topic was deemed important and your approaches seem reasonable so we would like to give you the opportunity to submit a revised version. Please take into account the reviewers comments and be sure to reorganize your manuscript in a logical manner that is easy to follow, even for people who are not in the lipid/lipid transport field and who do not work with yeast.
I hope that you are able to do that. Please include a point by point response to the referees in your revised version. I will decide whether or not the manuscript needs to go out for re-review when I see the revised version and the changes you have done.
Best regards,
Howard Riezman

Sincerely,

Howard Riezman
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Kishimoto,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office

mbc@ascb.org

Reviewer #1 (Remarks to the Author):

This is an unwieldy paper, presenting an enormous amount of data. The data are of high quality. However, they are presented in such abundance and almost with no selection that the only way to negotiate the main logical thread of the paper is to refer constantly to the abstract. I considered whether the paper could be divided into two papers but could not find an easy way to do this.

I have some points for the authors to address.
1. line 61: add reference on transbilayer lipid asymmetry (Kobayashi, Menon (2018) Curr Biol)
2. Fig 1A: it is not immediately clear from the figure that the plasmids are non-URA3, and that the strains carry pRS315-SFK1; this can be discovered in the fig legend, but there is some much to read here that it would be easier for the authors to make this point obvious in the figure itself.
3. line 163: why is a triple GFP tag used? this seems unnecessarily large.
4. Fig 2F and lines 210-215: the authors note the loss of PM staining with the PS sensors, but do not comment on the intracellular accumulation of the sensors.
5. Fig 3B: the sucrose gradient separation is quite poor. See better data in Quon 2018, Georgiev 2011
6. line 247: for the future, the authors could consider using Con A-mediated isolation of the PM
7. line 253: it is strange to find Can1 at the PM in rich medium - under these conditions, the transporter should be in internal membranes.
8. lines 300-301: the authors should be more circumspect about this conclusion. The defect could be in sterol transport OR homeostasis/distribution in the cell
9. line 316: how is it concluded that the triple mutant may have a defect in ergosterol biosynthesis?
10. line 361: ERG11 shut-off should stall cell growth, not cause loss of ergosterol content per cell. With this in mind, the sensor should bind equally well after ERG11 shutoff. The authors should explain this result a bit more.
11. Solanko 2018 present data showing that ergosterol is localized to the cytoplasmic leaflet of the PM, and that its transbilayer distribution can be affected by acute alteration of sphingolipid synthesis with myriocin. The authors could discuss the implications of these results in the context of their work.

Reviewer #2 (Remarks to the Author):

Phospholipid flippases and Sfk1 are essential for the retention of ergosterol in the 2 plasma membrane
The manuscript aims at understanding the role of P4-ATPase dnf3, non-catalytic subunit crf1 and the protein Sfk1 in regulating membrane lipid asymmetry and composition.

The major findings include:
1. The deletion of crf1 in the background of lem3 and sfk1 null leads to loss of PS and PE from the cytosolic leaflet of the plasma membrane along with defects in cell growth and certain membrane properties such as its density and permeability.
2. Under the same conditions there is a significant loss of ergosterol from the plasma membrane which is later localized to lipid droplets inside the cells as esterified ergosterol. This cannot be rescued by external addition of ergosterol.
3. Inhibition of esterification of ergosterol or overexpression of KES1 which is a sterol binding protein is able to rescue some of the defects
4. SFK1 and 'active' or 'accessible' ergosterol have an anti-correlation in their expressions between mother and daughter cells in budding yeast thereby implicating SKF1 in playing an antagonistic role in regulating 'active' cholesterol levels.

While the general idea of loss of lipid asymmetry and perturbation of ergosterol levels upon suppressing P4-ATPases have been studied earlier, including by these authors, the effects of dnf3-crf1 complex on this steady state have been explored for the first time here.

There is a potential of presenting a novel set of observations and ideas as the complex studied here is a TGN localized flippase rather than PM localized. The roles of PM-localized flippases have been explored by the authors earlier as well as the relationship of the function of the flippases/ floppases and skf1 proteins in mediating 'active' cholesterol homeostasis.

However several caveats remain:

General:
1) Without significant emphasis on the role of the TGN localized flippase (the new observation in a series of papers from the author (see in particular Mioka et al, 2018), the current manuscript appears to only provide an incremental extension of the

observations from the previous mutations.

2) There is a lack of a binding hypothesis or a working model to explain the observations presented here which makes a lot of these observations appear acontextual, and redundant with the earlier work.

Specifically:
1. How do the authors explain that most phenotypes are expressed only in the triple mutant and not the double mutants? For eg. growth defect, PE exposure, especially PS exposure etc.

2. What is the working model for explaining the growth defects particularly in the triple mutants which show a significant loss of ergosterol? What is the role of loss of asymmetry in this process? Could PS be playing a role through its charge and head group size in determining the curvature of the respective leaflet and consequent formation of irregular structures in the cytosol? Hence, with the lack of flipping of PS is there a potential reduction in formation of exocytic vesicles from the TGN where dnf3-crf1 is localized leading to further downstream consequences at PM? Is it possible to show with a loss of function mutations specific to the PS, PE flipping roles of dnf3-crf1 and its rescue or gain of function mutations in other flippase complexes and its rescue that the observed phenotypes are indeed due to loss of asymmetry?

3. Dnf3 is TGN localized. How can one explain that it mostly affects the plasma membrane ergosterol rather than the ER or TGN content? Would it be possible to explore the levels of those? Is it possible that the irregular ER structures seen are due to dnf3 based effects on lipid levels in the TGN and membrane tension?

4. Given that trafficking is unaffected, would it be possible to explain the same phenotype considering sorting defects post synthesis of these proteins which could lead to an overall imbalance in membrane trafficking and sorting.

For eg in the article by Hankins et al, MBoC, 2015, PS flipping has been proposed to play a role in sorting of proteins at the TGN. The idea being that PS when flipped to the cytosolic leaflet leads to exchange of ergosterol to the lumenal leaflet which when present in high concentrations leads to nucleation of microdomains where proteins can preferentially segregate finally concentrating in exocytic vesicles rather than the endocytic system. In the absence of PS flipping this network falls apart leading to miss sorting of proteins and their presence in internal organs probably through higher association with the endocytic system. This does not get affected by perturbation of any other modes of trafficking. Could something similar be going on in these deletions as well?

5. Noting that the interactions between the P4 ATPases and cdc50 like subunits is necessary not only for lipid translocation but for proper exit from ER and accurate subcellular localization and that dnf3-crf1 is an TGN localized complex could the observed phenotypes be because of loss of this interaction? There is a difference in subcellular localisation of dnf3 and crf1 when endocytosis is inhibited (fig 1E). However, it is not convincing that this result implies that this leads to a defect in the plasma membrane, which in turn result in the cell growth defects in the triple mutant strain. The WT does not show almost any PM localization of the two subunits.

6. The mechanism of Kes1 overexpression that partially rescues the growth defect in the triple mutant is explained by the ability of Kes1 to RAPIDLY transfer 'active' cholesterol back to the PM, and prevent it from accumulating in the ER to become esterified and form lipid droplets. If this is the case over expression of Kes1 in wild type cells should in turn equalize 'active' cholesterol levels between the PM and the ER, thereby should create a similar growth defect. The role of Kes1 is puzzling in the absence of a hypothesis of its function in wild type cells and its relationship to the function of skf1.

7. The direct role of SFK1 has not been addressed. How does SFK1 interacts with ergosterol to make it 'inaccessible' to D4H? What would be the role of lipid shielding (umbrella model) in that case? Since it has been suggested that sfk1 does not play a role in regulating ergosterol levels (Mioka et al, MBoC, 2018) is it possible that Sfk1 is actually regulating the 'accessibility' of cholesterol (rather than levels) which increases upon loss of phospholipid based shielding and is recognized by Kes1 and deposited in structures inside the cell? Can you follow sfk1 signal with respect to PE or PS exposure signals apart from active sterol signals to understand whether the daughter cell I sreally symmetric or otherwise? Since both Kes1 overexpression(this manuscript) and sfk1 overexpression (Mioka et al, 2018) leads to rescue of asymmetry based phenotype they might be acting on the same component namely active ergosterol? Further, if the claim is daughter cells are not asymmetric in composition but however loss of asymmetry leads to growth defect, then how do these daughter cells bud or grow?

8. In the presence of flippases, lipid flipping occurs at much faster timescales (Pomorski and Menon, PLR, 2017) compared to that of the experiment carried out here. How would this explain the daughter cells to be symmetric in composition? Is there a delay in activating flippases activity in the daughter cell, or are flippases not transported to the daughter cells. Can the daughter cell bud be followed to ascertain when establishment of asymmetry through PE or PS signals and ergosterol signals is correlated with specific protein localization?

9. While fig 3B shows that membrane density reduces, the follow up fact that the transporters are not localized to the PM as accurately as in the WT doesn't necessarily imply that the membrane integrity is lost or that it is the cause of this effect (fig 3C,D). Correspondingly, does the vrp1 mutant line which is also endocytosis deficient corroborate the results seen upon Lat

treatment?

10. The experiment using biotin R0-0198 is a nice experiment which indicates that PE is indeed more exposed in triple mutants. Why does the crf1Δsfk1Δ not expose PE? The function of the dnf3 should be inhibited in this mutant as well.

Other

1. Can the quantifications be done not just as % of cells showing an effect but the extent of change of a parameter as has been done in figure 2E. This might help address if there is a threshold in lipid levels such as ergosterol that is reached before showing the growth defects in the triple mutants.

2. In order to directly establish that the triple mutants are indeed more sensitive to duramycin because of higher PE exposure is it possible to do a dose response curve starting from lower concentrations of duramycin compared to the concentration used in the study.

3. It might be useful to tabulate changes in parameters (growth rate, lipid exposure, sterol level change etc.) across each mutant (single, double and triple) to understand any emerging patterns in role of each subunit.

We wish to express our appreciation to the reviewers for their insightful comments on our paper, which have helped us significantly improve the paper. We are grateful for their spending precious time and energy on reviewing our paper.

Reviewer #1 (Remarks to the Author):

This is an unwieldy paper, presenting an enormous amount of data. The data are of high quality. However, they are presented in such abundance and almost with no selection that the only way to negotiate the main logical thread of the paper is to refer constantly to the abstract. I considered whether the paper could be divided into two papers but could not find an easy way to do this.

We thank the reviewer for this important comment. We thought that those data may be informative to readers, but we agree with the reviewer's comment that those data interrupt the main logical thread of the paper. The main conclusion of our paper is that the simultaneous loss of LEM3, SFK1, and CRF1 results in defects in retention of ergosterol in the plasma membrane. Accordingly, we have deleted the results of the localization of Sfk1-2 mutant protein (Fig. 2C in the original version), the mislocalization of amino acid transporters (Fig. 3C and D, Fig. 4C, and Supp Fig. 4), the localization of Kes1 to abnormal ER structures (Fig. 4 E, F, and G and Supp Fig. 6), the localization of Osh2-PH and growth phenotype of the sfk1ΔC mutant (Supp Fig. 5), and the correlation between the triple mutant and triacylglycerol (Supp Fig. 12).

I have some points for the authors to address.
1. line 61: add reference on transbilayer lipid asymmetry (Kobayashi, Menon (2018) Curr Biol)

We thank the reviewer for the suggestion. The suggested reference has been added to the revised version (line 60).

2. Fig 1A: it is not immediately clear from the figure that the plasmids are non-URA3, and that the strains carry pRS315-SFK1; this can be discovered in the fig legend, but there is some much to read here that it would be easier for the authors to make this point obvious in the figure itself.

We thank the reviewer for the comment. According to the reviewer's suggestion, we have added a drawing to Fig. 1A to explain the experiment. In addition, according to the editor's

suggestion, we have also added a drawing to the tetrad data to explain the tetrad analysis for people who do not work with yeast. The results of tetrad analysis have been moved to Supp Fig. 1 in the revised version.

3. line 163: why is a triple GFP tag used? this seems unnecessarily large.

We used DNF3-3xGFP, because the signal intensity of DNF3-GFP was very weak. We confirmed that it is functional, because the *lem3*Δ *sfk1*Δ mutant containing DNF3-3xGFP or CRF1-GFP grew normally. This has been described in the revised version (lines 563 to 565).

4. Fig 2F and lines 210-215: the authors note the loss of PM staining with the PS sensors, but do not comment on the intracellular accumulation of the sensors.

As pointed out by the reviewer, we should comment on the intracellular accumulation of the sensors. Both GFP-Lact-C2 and GFP-evt-2PH were localized to intracellular structures in the triple mutant. However, they appeared to be localized to different structures, which may represent PS-containing membranes or nonspecific protein aggregations. These sentences have been added to the revised version (lines 212 to 215).

5. Fig 3B: the sucrose gradient separation is quite poor. See better data in Quon 2018, Georgiev 2011

According to the reviewer's suggestion, we have performed the sucrose gradient fractionation according to the procedures by Georgiev et al., 2011, with minor modifications. The fractionation pattern has been improved. Thus, Fig. 3B of the original version has been replaced with the new Fig. 3B in the revised version.

6. line 247: for the future, the authors could consider using Con A-mediated isolation of the PM

We thank the reviewer for the valuable suggestion. We would like to attempt to perform the experiment in our next study.

7. line 253: it is strange to find Can1 at the PM in rich medium - under these conditions, the transporter should be in internal membranes.

In our strain background, the Can1-GFP signal was also observed in the PM in addition to the vacuole in YPDA rich medium. However, according to the reviewer's suggestion, the results containing localizations of amino acid transporters have been deleted in the revised version, because these results are not closely related to the main story.

8. lines 300-301: the authors should be more circumspect about this conclusion. The defect could be in sterol transport OR homeostasis/distribution in the cell

We totally agree to the reviewer's comment. According to the reviewer's suggestion, we have changed the sentence as follows. "These results suggest that the *crf1*Δ *lem3*Δ *sfk1-2* triple mutant may have a defect in intracellular transport, homeostasis, or distribution of ergosterol." (lines 272 to 273)

9. line 316: how is it concluded that the triple mutant may have a defect in ergosterol biosynthesis?

In the original version, it is ergosterol "homeostasis", not "biosynthesis", although this part has been deleted in the revised version according to the reviewer's suggestion.

10. line 361: ERG11 shut-off should stall cell growth, not cause loss of ergosterol content per cell. With this in mind, the sensor should bind equally well after ERG11 shutoff. The authors should explain this result a bit more.

We have performed the experiment to examine the effect of ERG11 shut-off on the ergosterol level. As shown in Supp Fig. 6A, the free ergosterol level decreased to 60~70 % when the expression of ERG11 was repressed. This has been described in the revised version (lines 313 to 315).

11. Solanko 2018 present data showing that ergosterol is localized to the cytoplasmic leaflet of the PM, and that its transbilayer distribution can be affected by acute alteration of sphingolipid synthesis with myriocin. The authors could discuss the implications of these results in the context of their work.

In Solanko et al., 2018, it was shown that sphingolipids are required to maintain ergosterol in the cytoplasmic leaflet. Why ergosterol is abundant in the cytoplasmic leaflet is an interesting question. However, this is not closely related to the main topic of our paper, which is the ergosterol activation in the cytoplasmic leaflet. We would like to investigate ergosterol

asymmetry in our future work, in which the finding by Solanko et al. would be discussed.

Reviewer #2 (Remarks to the Author):

Phospholipid flippases and Sfk1 are essential for the retention of ergosterol in the 2 plasma membrane
The manuscript aims at understanding the role of P4-ATPase dnf3, non-catalytic subunit crf1 and the protein Sfk1 in regulating membrane lipid asymmetry and composition.
The major findings include: 1. The deletion of crf1 in the background of lem3 and sfk1 null leads to loss of PS and PE from the cytosolic leaflet of the plasma membrane along with defects in cell growth and certain membrane properties such as its density and permeability.
2. Under the same conditions there is a significant loss of ergosterol from the plasma membrane which is later localized to lipid droplets inside the cells as esterified ergosterol. This cannot be rescued by external addition of ergosterol.
3. Inhibition of esterification of ergosterol or overexpression of KES1 which is a sterol binding protein is able to rescue some of the defects
4. SFK1 and 'active' or 'accessible' ergosterol have an anti-correlation in their expressions between mother and daughter cells in budding yeast thereby implicating SKF1 in playing an antagonistic role in regulating 'active' cholesterol levels.
While the general idea of loss of lipid asymmetry and perturbation of ergosterol levels upon suppressing P4-ATPases have been studied earlier, including by these authors, the effects of dnf3-crf1 complex on this steady state have been explored for the first time here.
There is a potential of presenting a novel set of observations and ideas as the complex studied here is a TGN localized flippase rather than PM localized. The roles of PM-localized flippases have been explored by the authors earlier as well as the relationship of the function of the flippases/ floppases and skf1 proteins in mediating 'active' cholesterol homeostasis.

However several caveats remain:

General:
1) Without significant emphasis on the role of the TGN localized flippase (the new observation in a series of papers from the author (see in particular Mioka et al, 2018), the current manuscript appears to only provide an incremental extension of the observations from the previous mutations.

We appreciate the reviewer's comments. As pointed out by the reviewer, we should also put emphasis on the fact that Dnf3-Crf1 is mainly localized to the TGN. According to the reviewer's suggestion, we have described the possibility that the Dnf3-Crf1 localized in the TGN regulates phospholipid asymmetry in the PM (lines 442-445). However, we cannot exclude the possibility that a small population of Dnf3-Crf1 in the PM coordinately functions with Dnf1/2-Lem3 and Sfk1. Even in the latter case, our observations are not incremental extension of those in Mioka et al. (2018), in which only the lem3 sfk1 double mutant was analyzed. We included the results of the lem3 sfk1-2 double mutant in Supplemental Figures as control experiments in the original version. There are clear phenotypic differences between the lem3 sfk1-2 double and crf1 lem3 sfk1-2 triple mutants; for example, in the revised version, filipin and GFPenvy-D4H stain the PM in the lem3 sfk1-2 mutant (Supp Fig. 5 and Supp Fig. 6D), but not in the crf1 lem3 sfk1-2 mutant (Fig. 5A and F). TopFluor-cholesterol is retained in the PM in the lem3 sfk1-2 upc2-1 mutant (Supp Fig. 7), but not in the crf1 lem3 sfk1-2 upc2-1 mutant (Fig. 6B). Lipid droplets were clearly accumulated in the triple mutant (Fig. 7C), but not in the double mutant (Supp Fig. 9). In Mioka et al. (2018), we showed that permeability of the PM is increased, and that "total ergosterol" is somewhat decreased in the lem3 sfk1 mutant, but we did not observe a defect in the retention of ergosterol in the PM. Therefore, irrespective of the localization of Dnf3-Crf1 in the TGN or the PM, our current results indicate that Crf1, Lem3, and Sfk1 coordinately play an essential role in the retention of ergosterol in the PM.

2) There is a lack of a binding hypothesis or a working model to explain the observations presented here which makes a lot of these observations appear acontextual, and redundant with the earlier work.

Our working model is that flippases and Sfk1 coordinately function to retain ergosterol in the PM. Although biochemical functions of Sfk1 remain unknown, it might suppress active ergosterol in the cytoplasmic leaflet of the PM as suggested in Fig. 9. We then discuss how phospholipid asymmetry and Sfk1 could retain ergosterol in the PM. Our model has been depicted in Fig. 10 in the revised version.

As also pointed out by the reviewer #1, the original version contains the results that may not be closely related to the main story. According to the reviewer #1 and #2s' comments, we have deleted those results in the revised version. As to the deleted figures, please see our response to the first comment of the reviewer #1. As described in our response to the comment #1, the revised version focuses on the phenotypes of the crf1 lem3 sfk1 mutant, including the defects of ergosterol retention in the PM, which is not described in Mioka et al., 2018.

Specifically:
1. How do the authors explain that most phenotypes are expressed only in the triple mutant and not the double mutants? For eg. growth defect, PE exposure, especially PS exposure etc.

A major finding in this work is that there is functional redundancy among Dnf1/2-Lem3, Sfk1, and Dnf3-Crf1. Most important phenotypes, including growth defects and loss of ergosterol from the PM, are only found in the triple mutant, not in double mutants in any combination. Because synthetic genetic interactions are occasionally observed between structurally unrelated genes (Costanzo M, et al., Science 327:425-431, 2010), a triple synthetic genetic interaction would not be surprising. Importantly, two flippases and another type of protein, Sfk1, play an essential role in retaining sterols in the PM. This suggests that yeast cells have acquired a robust system to retain an important molecule, ergosterol, in the PM. This has been discussed in the revised version (lines 517-518).

2. What is the working model for explaining the growth defects particularly in the triple mutants which show a significant loss of ergosterol? What is the role of loss of asymmetry in this process? Could PS be playing a role through its charge and head group size in determining the curvature of the respective leaflet and consequent formation of irregular structures in the cytosol? Hence, with the lack of flipping of PS is there a potential reduction in formation of exocytic vesicles from the TGN where dnf3-crf1 is localized leading to further downstream consequences at PM? Is it possible to show with a loss of function mutations specific to the PS, PE flipping roles of dnf3-crf1 and its rescue or gain of function mutations in other flippase complexes and its rescue that the observed phenotypes are indeed due to loss of asymmetry?

The growth defect could be due to increased permeability of the PM or abnormal regulation or/and function of PM proteins. This has been added to the revised version (lines 438-439). As to why loss of phospholipid asymmetry causes the loss of ergosterol from the PM, it had been discussed in the original version (lines 446 to 461 in the revised version); the PM is rich in the PS and PE species containing saturated acyl chains, which favorably interact with sterols. In addition, PS with a large headgroup has a higher affinity for cholesterol. Therefore, loss of PS and PE asymmetry would result in an increase in “active ergosterol” in the cytoplasmic leaflet, which would be actively removed by sterol transfer proteins. As discussed in the revised version (lines 442-445), Dnf3-Crf1 could control phospholipid asymmetry of the PM through transport of TGN-derived vesicles. As to substrate specificity

and effects of a PS- or PE-specific mutant of Dnf3, because flippase activity has not been demonstrated for Dnf3-Crf1, we would like to investigate them in our future study.

3. Dnf3 is TGN localized. How can one explain that it mostly affects the plasma membrane ergosterol rather than the ER or TGN content? Would it be possible to explore the levels of those? Is it possible that the irregular ER structures seen are due to dnf3 based effects on lipid levels in the TGN and membrane tension?

Loss of ergosterol from the PM and irregular ER structures are observed only when the dnf3 mutation was combined with the mutations of Lem3 and Sfk1, which are PM proteins. How Dnf3-Crf1 in the TGN could affect phospholipid asymmetry in the PM has been discussed in the revised version (lines 442-445). The dnf3 single mutant does not show any clear phenotype, including irregular ER structures.

4. Given that trafficking is unaffected, would it be possible to explain the same phenotype considering sorting defects post synthesis of these proteins which could lead to an overall imbalance in membrane trafficking and sorting.

For eg in the article by Hankins et al, MBoC, 2015, PS flipping has been proposed to play a role in sorting of proteins at the TGN. The idea being that PS when flipped to the cytosolic leaflet leads to exchange of ergosterol to the lumenal leaflet which when present in high concentrations leads to nucleation of microdomains where proteins can preferentially segregate finally concentrating in exocytic vesicles rather than the endocytic system. In the absence of PS flipping this network falls apart leading to miss sorting of proteins and their presence in internal organs probably through higher association with the endocytic system. This does not get affected by perturbation of any other modes of trafficking. Could something similar be going on in these deletions as well?

We thank the reviewer for pointing out this possibility. The crf1/dnf3 mutation could lead to missorting of an unknown PM protein that regulates phospholipid asymmetry of the PM. This possibility has been discussed in the revised version (lines 444-445).

5. Noting that the interactions between the P4 ATPases and cdc50 like subunits is necessary not only for lipid translocation but for proper exit from ER and accurate subcellular localization and that dnf3-crf1 is an TGN localized complex could the observed phenotypes be because of loss of this interaction? There is a difference in subcellular localisation of dnf3 and crf1 when endocytosis is inhibited (fig 1E). However, it is not convincing that this result

implies that this leads to a defect in the plasma membrane, which in turn result in the cell growth defects in the triple mutant strain. The WT does not show almost any PM localization of the two subunits.

Because both the crf1 and dnf3 mutations exhibited synthetic lethality with the lem3 sfk1 mutations (Supp Fig. 1), the observed phenotypes are because of loss of this flippase, not specifically because of loss of the protein interaction between Dnf3 and Crf1.

As pointed out by the reviewer, we could not detect either Dnf3-GFP or Crf1-GFP in the PM in wild-type cells. However, the results of the vrp1 mutant suggest that Dnf3-Crf1 is recycled between the PM and endosome/TGN. In our revised version, we discuss that Dnf3-Crf1 would coordinately function with Dnf1/2-Lem3 and Sfk1 at the TGN or/and PM (lines 439-445). In either case, Dnf3-Crf1 plays an essential role in retaining ergosterol in the PM in conjunction with Dnf1/2-Lem3 and Sfk1, which is a main finding of our paper.

6. The mechanism of Kes1 overexpression that partially rescues the growth defect in the triple mutant is explained by the ability of Kes1 to RAPIDLY transfer 'active' cholesterol back to the PM, and prevent it from accumulating in the ER to become esterified and form lipid droplets. If this is the case over expression of Kes1 in wild type cells should in turn equalize 'active' cholesterol levels between the PM and the ER, thereby should create a similar growth defect. The role of Kes1 is puzzling in the absence of a hypothesis of its function in wild type cells and its relationship to the function of skf1.

Although the involvement of Kes1 in sterol homeostasis, including ergosterol transport, seems to be established, its precise transport mechanisms and transport routes remain to be explored, as suggested by the reviewer. The suppression of the growth defects by Kes1 overexpression in the crf1 lem3 sfk1 mutant provided us a clue to defects that occur in the triple mutant, which led us to analyze ergosterol distribution. According to the editor's and reviewers' suggestions, we have deleted the results that are not closely related to the main story. Accordingly, we have deleted the results of localization of Kes1 to abnormal ER structures in the revised version. Thus, in the revised version, we have deleted our proposal that Kes1 overexpression suppresses the growth defect by transporting ergosterol from the ER to the PM (Discussion, lines 553-556 in the original version).

7. The direct role of SFK1 has not been addressed. How does SFK1 interacts with ergosterol to make it 'inaccessible' to D4H? What would be the role of lipid shielding (umbrella model) in that case? Since it has been suggested that sfk1 does not play a role in regulating ergosterol levels (Mioka et al, MBoC, 2018) is it possible that Sfk1 is actually regulating the

'accessibility' of cholesterol (rather than levels) which increases upon loss of phospholipid based shielding and is recognized by Kes1 and deposited in structures inside the cell? Can you follow sfk1 signal with respect to PE or PS exposure signals apart from active sterol signals to understand whether the daughter cell I sreally symmetric or otherwise? Since both Kes1 overexpression(this manuscript) and sfk1 overexpression (Mioka et al, 2018) leads to rescue of asymmetry based phenotype they might be acting on the same component namely active ergosterol? Further, if the claim is daughter cells are not asymmetric in composition but however loss of asymmetry leads to growth defect, then how do these daughter cells bud or grow?

As pointed out by the reviewer, we have not addressed the direct role of Sfk1. Because biochemical experiments are required for this purpose, we would like to investigate it in our future work. As suggested by the reviewer, we presume that Sfk1 negatively regulates accessibility to ergosterol for ergosterol-interacting proteins. Our model has been depicted in Fig. 10 in the revised version. As discussed in the original and revised versions (lines 487-488), Sfk1 may enhance interactions between ergosterol and phospholipids, and thus, Sfk1 decreases active ergosterol.

We are not proposing that phospholipids are symmetrically distributed across the bilayer in daughter cells. PE asymmetry is apparently generated by the Dnf1/Dnf2-Lem3 flippase in daughter cells, because the biotinylated Ro peptide does not bind to the daughter cell PM in wild-type cells (Iwamoto et al., Genes to Cells, 9:891-903, 2004). When the daughter cell PM is expanded by vesicle fusion, PE and PS would be exposed, but would be rapidly flipped to the cytoplasmic leaflet by Dnf1/2-Lem3. We have revised the sentence to avoid confusion in the revised version (lines 478-483). Our proposal is that the daughter cell PM contains more active ergosterol, probably because phospholipid asymmetry has not been "completely" established

8. In the presence of flippases, lipid flipping occurs at much faster timescales (Pomorski and Menon, PLR, 2017) compared to that of the experiment carried out here. How would this explain the daughter cells to be symmetric in composition? Is there a delay in activating flippases activity in the daughter cell, or are flippases not transported to the daughter cells. Can the daughter cell bud be followed to ascertain when establishment of asymmetry through PE or PS signals and ergosterol signals is correlated with specific protein localization?

As described in our responses to the comment #7, phospholipid asymmetry is generated in the daughter cell PM by flippases, although it may not be completely established. What we found is that the daughter cell PM contains more active ergosterol compared to the mother cell PM,

but this does not indicate that phospholipid asymmetry is not generated. Lipids and proteins are being organized in the PM of daughter cells during budding, suggesting that the daughter cell PM is immature compared to the mother cell PM.

9. While fig 3B shows that membrane density reduces, the follow up fact that the transporters are not localized to the PM as accurately as in the WT doesn't necessarily imply that the membrane integrity is lost or that it is the cause of this effect (fig 3C,D). Correspondingly, does the vrp1 mutant line which is also endocytosis deficient corroborate the results seen upon Lat treatment?

According to the editor's and reviewers' suggestions, we have deleted the figures that are not closely related to the main story in the revised version, including the mislocalization of amino acid transporters in the triple mutant (Fig. 3C and D and Supp Fig. 4 in the original version).

10. The experiment using biotin R0-0198 is a nice experiment which indicates that PE is indeed more exposed in triple mutants. Why does the crf1Δsfk1Δ not expose PE? The function of the dnf3 should be inhibited in this mutant as well.

It appears that Dnf1/2-Lem3 mainly functions to repress PE and PS exposure. When single mutants are compared, the lem3 mutant showed strongest phenotypes in biotinylated Ro 09-0198 staining and papuamide B and duramycin sensitivity. The contributions by Crf1 and Sfk1 may be minor, but they are enough for cell growth in the absence of Lem3.

Other
1. Can the quantifications be done not just as % of cells showing an effect but the extent of change of a parameter as has been done in figure 2E. This might help address if there is a threshold in lipid levels such as ergosterol that is reached before showing the growth defects in the triple mutants.

We thank the reviewer for this comment. However, in the case of GFPenvy-D4H, the signal intensity does not equally reflect ergosterol level among the individual cells, because GFPenvy-D4H is expressed from a centromeric plasmid. We constructed a strain in which GFPenvy-D4H was integrated into the genome, but the signal was not strong enough for an unknown reason. We would like to perform the suggested experiments when we have constructed a suitable strain.

2. In order to directly establish that the triple mutants are indeed more sensitive to duramycin

because of higher PE exposure is it possible to do a dose response curve starting from lower concentrations of duramycin compared to the concentration used in the study.

According to the reviewer's suggestion, we have performed the dose-response growth curve experiment to duramycin. As shown in Supp Fig. 3B in the revised version, the crf1 lem3 sfk1-2 triple mutant exhibited the highest sensitivity to duramycin (liens 191-192).

3. It might be useful to tabulate changes in parameters (growth rate, lipid exposure, sterol level change etc.) across each mutant (single, double and triple) to understand any emerging patterns in role of each subunit.

We thank the reviewer for the suggestion. As described in our response to the comment #10, Dnf1/2-Lem3 plays a major role in generating phospholipid asymmetry in the PM compared to Sfk1 and Dnf3-Crf1. Thus, double mutants containing the lem3 mutation showed some phenotypes (e.g. PE exposure in Fig. 2D), but not in other experiments (e.g. GFP-evt-2PH localization in Fig. 2E). We only noticed some weak phenotypes in lem3-containing double mutants, which are shown in each figure. Except for those phenotypes, any single or double mutant did not show a significant phenotype. Therefore, we have not shown those results in a table in the revised version.

RE: Manuscript #E20-11-0699R
TITLE: "Phospholipid flippases and Sfk1 are essential for the retention of ergosterol in the plasma membrane"

Dear Dr Kishimoto,
Your revised manuscript has now been seen by the two original reviewers, who agree that the manuscript has been greatly improved and now merits publication. There is only one minor comment on the text brought up by reviewer 2 that you might want to deal with to ensure the best clarity of presentation. Once we receive your response/revised version I will quickly take the final decision.
Best regards,
Howard Riezman

Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Kishimoto,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

no comments

Reviewer #2 (Remarks to the Author):

The revised manuscript is a significant improvement on the meandering original. It now identifies central question(s) and addresses them with a defined set of experiments with clear outcomes. This is attributed both to revision in the text and in the flow of the final figures.
However, the speculation/explanation about the difference in GFPenvy-D4 staining in the daughter-mother cell membrane asymmetry remains difficult to understand. In the view of this reviewer, the relevance of this aspect to the overall core of this manuscript is somewhat ambiguous. If the authors feel that this aspect must remain it would be good to link up this section with a discussion on how asymmetry arises in the growing daughter bud. Overall the authors have made a good attempt at improving the manuscript and their arguments. Their arguments regarding the mechanism of concentration of ergosterol related to the asymmetry of phospholipid distribution in the membrane, are convincing.

Reviewer #2 (Remarks to the Author):

The revised manuscript is a significant improvement on the meandering original. It now identifies central question(s) and addresses them with a defined set of experiments with clear outcomes. This is attributed both to revision in the text and in the flow of the final figures. However, the speculation/explanation about the difference in GFPenvy-D4 staining in the daughter-mother cell membrane asymmetry remains difficult to understand. In the view of this reviewer, the relevance of this aspect to the overall core of this manuscript is somewhat ambiguous. If the authors feel that this aspect must remain it would be good to link up this section with a discussion on how asymmetry arises in the growing daughter bud. Overall the authors have made a good attempt at improving the manuscript and their arguments. Their arguments regarding the mechanism of concentration of ergosterol related to the asymmetry of phospholipid distribution in the membrane, are convincing.

We thank the reviewer for important comments on our paper, which have helped us significantly improve our paper. We are thankful to the reviewer for his/her time and energy.

We totally agree with the reviewer #2's suggestions. As pointed out by the reviewer #2, we should explain more clearly the mechanisms that generate the differences in GFPenvy-D4H localizations observed between daughter and mother cells, i.e., those that regulate sterol activation. Since phospholipids, PS in particular, exhibit strong interactions with sterols, the asymmetry of phospholipids is thought to be closely related to sterol activation. Dnf1/2-Lem3 flippases are localized to the PM of daughter cells and flip phospholipids, which is expected to inhibit the sterol activation. However, since membrane biogenesis actively occurs through vesicle fusion, and since sterols might be also transported to the bud PM by sterol transfer proteins (STPs), sterols might not be fully embedded and might be exposed to the membrane surface, and thus we predict that GFPenvy-D4H detects them in the bud PM. In contrast, in the PM of the mother cell, membrane biogenesis would be less active and the phospholipid asymmetry has been established. We predict that there are mother cell-specific PM mechanisms that maintain the phospholipid asymmetry and control sterol activation, in which Sfk1 is involved. Sfk1 may repress spontaneous transbilayer movement of phospholipids in the PM of mother cell, which maintains sterols in an inactive state. These have been described in the Discussion of the second revised version (lines 480 to 498).

RE: Manuscript #E20-11-0699RR
TITLE: "Phospholipid flippases and Sfk1 are essential for the retention of ergosterol in the plasma membrane"

Dear Dr. Kishimoto:

Thank you for the final revised version of your manuscript and for all of your efforts. I am pleased to accept your manuscript on behalf of Molecular Biology of the Cell.

Best regards,
Howard Riezman

Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Kishimoto:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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